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We have proposed the identification of junctionally transmitted growth control signals which we hypothesize to be responsible for the growth inhibition of tumor cells when in junctional communication with normal cells. In order to test this hypothesis rigorously, we have genetically engineered human breast cancer cells to contain the gap junction gene connexin 43, under the control of a tetracycline inducible promoter. We have been successful in producing several clones in which connexin 43 is strongly induced on withdrawal of tetracycline and in which connexin 43 is integrated into the plasma membrane and is functional as determined by dye injection studies. We have moreover shown that exposure of isolated cells to EGTA results in the opening of hemi--channels to the extra-cellular environment; expression of connexin 43 is required for this effect. To determine that any responses of cells are due to junctional transfer of signals, rather than the presence of a trans-membrane protein, we have similarly produced breast cancer cell lines containing a mutant connexin 43 gene in which the pore is predicted to be blocked. These molecular and biological tools put us in a good position to carefully address the stated hypothesis .

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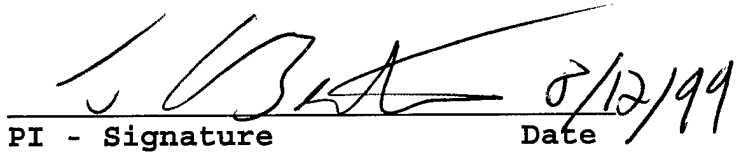

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Introduction:

We propose to chemically identify the junctionally transmitted signals which we hypothesize to be responsible for the observed growth inhibition of tumor cells when in junctional communication with growth inhibited normal cells.

Body:

Technical Objective 1a. Production of genetically engineered cells containing connexin 43 under the control of a tetracycline-inducible promoter. In order to maximize our chances of success, we have attempted the production of such engineered cells in to human breast cancer cell lines: MCF-7 and MDA. The first line was chosen because a commercially available cell line exists in which the tetracycline-receptor construct is already integrated; the second cell line was chosen because of prior reports that it was capable of expression and assembly of functional connexin 43.

Experience with MCF-7 cells: exhaustive attempts to produce antibiotic resistance cells, with or without co-transfection of the connexin 43 construct has routinely failed. Moreover, in our attempts to demonstrate a functional tetracycline receptor construct we have transfected these cells with a green fluorescent protein (GFP) construct under the control of the tetracycline promoter. This is a standard method for determining the presence of the tetracycline receptor. These studies to failed to demonstrate activation of this reporter gene, even though parallel transfections with B-gal yielded positive results. We found these cells were difficult to culture, had low growth rates and thus would be unsuitable for the propose studies. In communication with the commercial company from whom we purchase these cells it appears evident that success has not been achieved in other laboratories attempting to use as cell line. We have thus abandoned development of MCF-7 cell line as an inducible system. Because these MCF 7 cells proved inappropriate for the proposed studies, this has delayed the accomplishment of the most labor intensive portion of the Technical Objectives. However, as described below we have been successful with an alternate breast cancer cell line.

Experience with MDA cells: here we have achieved success in production of an inducible system for connexin 43 in which expression of the gene is suppressed by doxycycline and allowed on removal of doxycycline. Induction of this inducible line went through two stages: first the production of cells containing a tetracycline receptor stately integrated into the genome. In this phase of the research, approximately 80 antibiotic-resistant clones were isolated after transfection with the receptor and an antibiotic resistance marker, and these clones were tested for stable integration of this gene utilizing the transient transfection of a GFP plasmid in which the gene is under the control of a tetracycline promoter. Transfected cells were analyzed by fluorescence microscopsy, and those clones in which a substantial fraction of cells exhibited green fluorescence were selected for the second stage of the transfection procedure. We selected four clones for this purpose based on their ability to trans-activate the GFP gene, their ease of culture and consistent morphology.

The second stage of transfection utilized the connexin 43 construct and co-transfection with a second antibiotic resistance marker. This stage of the selection procedure was more difficult with the need to first identify cells exhibiting antibiotic resistance, second to determine which of these selected clones expressed connexin 43 at the protein level and in which this expression was doxycycline sensitive, finally these clones had to be assessed for their ability to assemble connexin 43 into functional gap junctional plaques. We have been successful in identifying five clones which are inducible for connexin 43 and in which this protein is functional. Data for one of these clones is

depicted in figures 1 and 2.

In Fig. 1 is shown a Western blot demonstrating that after withdrawal of doxycycline, a protein band, immunoreactive with a connexin 43 antibody and with a molecular mass of 43KD, is expressed in these cells. Moreover, as shown by the microinjection studies, induction of this gene converts these MDA cells from being junctionally non-competent to capable of rapidly transferring microinjected Lucifer yellow from a donor cell to approximately 30 adjacent cells. In the absence of induction, no junctional transfer was observed. Similar findings were obtained from approximately 30 microinjections. Consistent with this being due to be creation of gap junctions, immunofluorescent studies utilizing a connexin 43 antibody demonstrated that in induced cells, a network of immunoreactive junctional plaques was observed (Fig. 2). In contrast in the absence of induction, only background staining were seen. We thus conclude that we have successfully accomplished technical objective 1a, the creation of breast cancer cell lines made junctionally competent by transfection of a tet-inducible connexin gene.

Technical Objective 1b. Development of in vitro protocols for the delivery of the growth inhibitory signal from quiescent cells to junctionally-competent breast cancer cells. With the development of the inducible cell lines described above we are now situated to achieve this technical objective. Preliminary studies indicate that we are capable of opening hemi--channels in connexin 43 expressing MDA cells by exposure to low Ca++ medium. These results are shown in Fig. 3, in which the entry of Lucifer yellow is shown in cells briefly exposed to low Ca++ achieved by utilizing the Ca chelator EGTA. That this is not due to non-specific toxicity, is indicated by the absence of transfer of Lucifer yellow into cells in which connexin 43 was not induced. This lack of transfer of Lucifer yellow into the non-induced cells through hemi-channels utilizing low calcium conditions, or through intact gap junctions in the case of the microinjection studies shown in figure 1, all indicate that the MDA cells do not express functional junctions in the absence of gene induction.

Technical Objective 1c. Detection of the junctional transferred signal in breast cancer cells by measurement of cell cycle related parameters. These studies are currently underway. We are assessing the ability of induced cells to junctionally couple with growth arrested cells of fibroblast or epithelial origin and to become growth arrested when in this condition. We also determining whether, when junctionally coupled, these breast carcinoma cells can suppress their own proliferation. Results are not yet available from these studies.

Fig.1: Cx43 is inducible and functional in MDA cells as shown by Western immunoblotting and dye-transfer

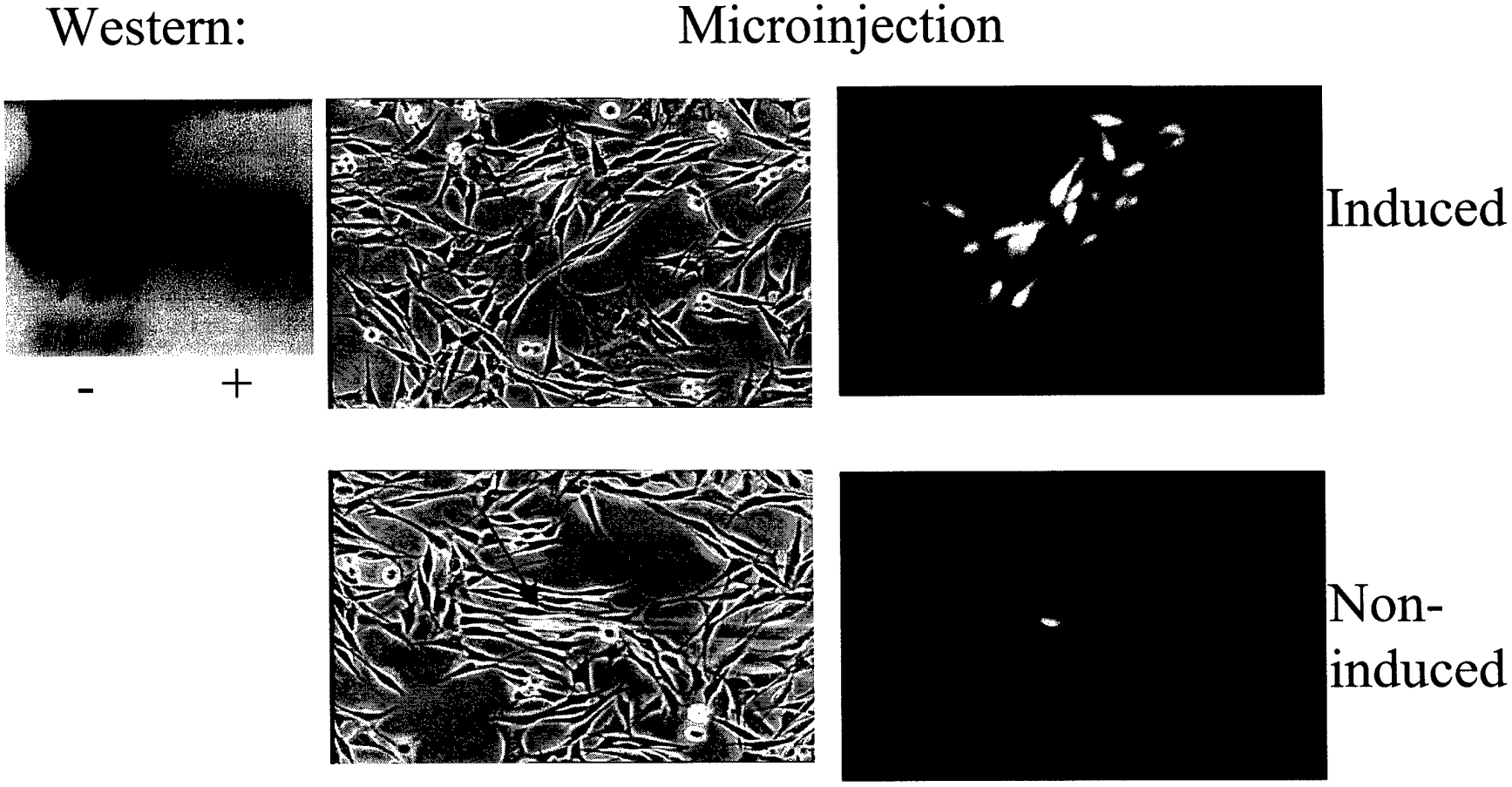


Fig. 2: Induced Cx43 is assembled into junctional plaques

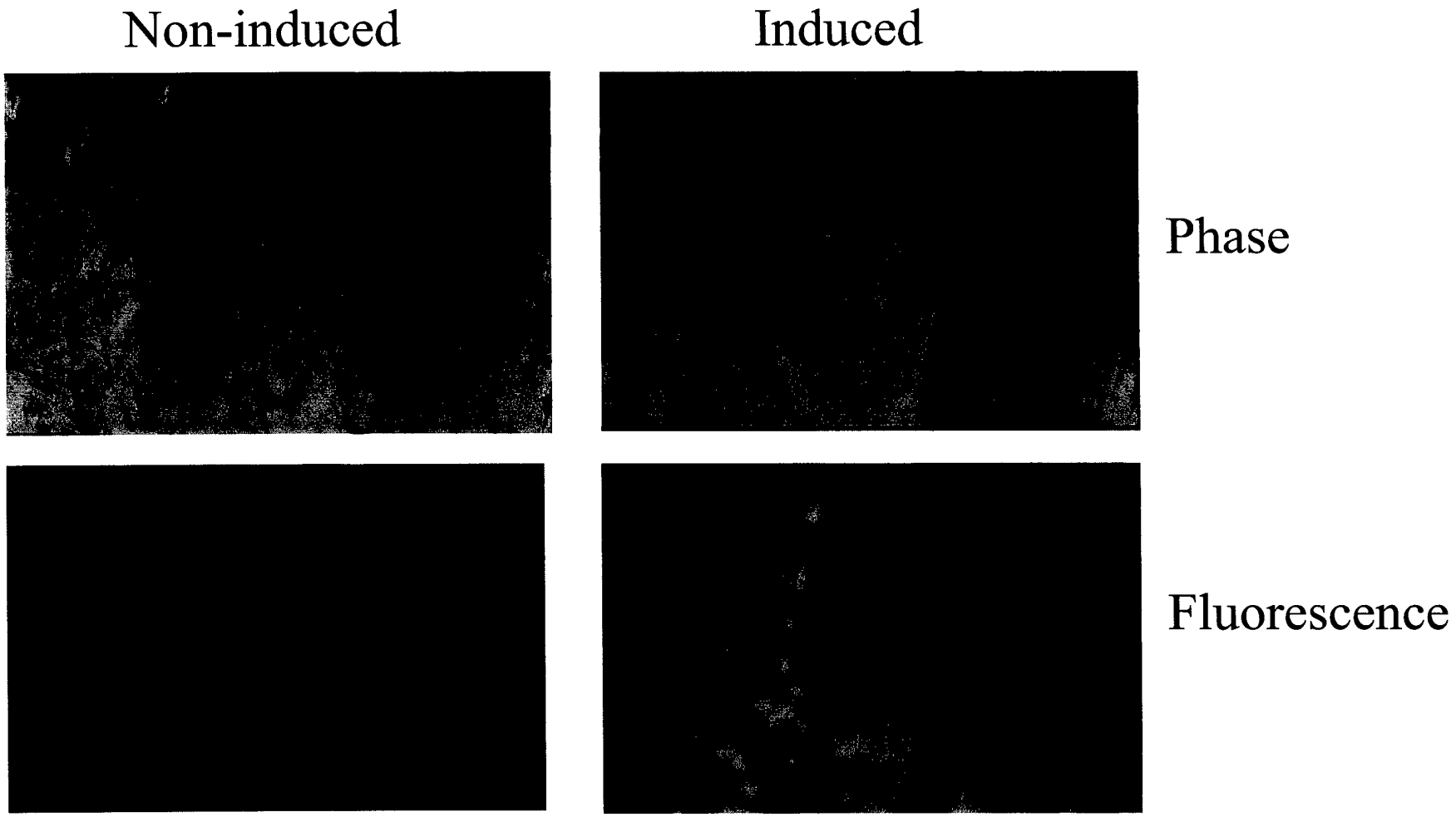
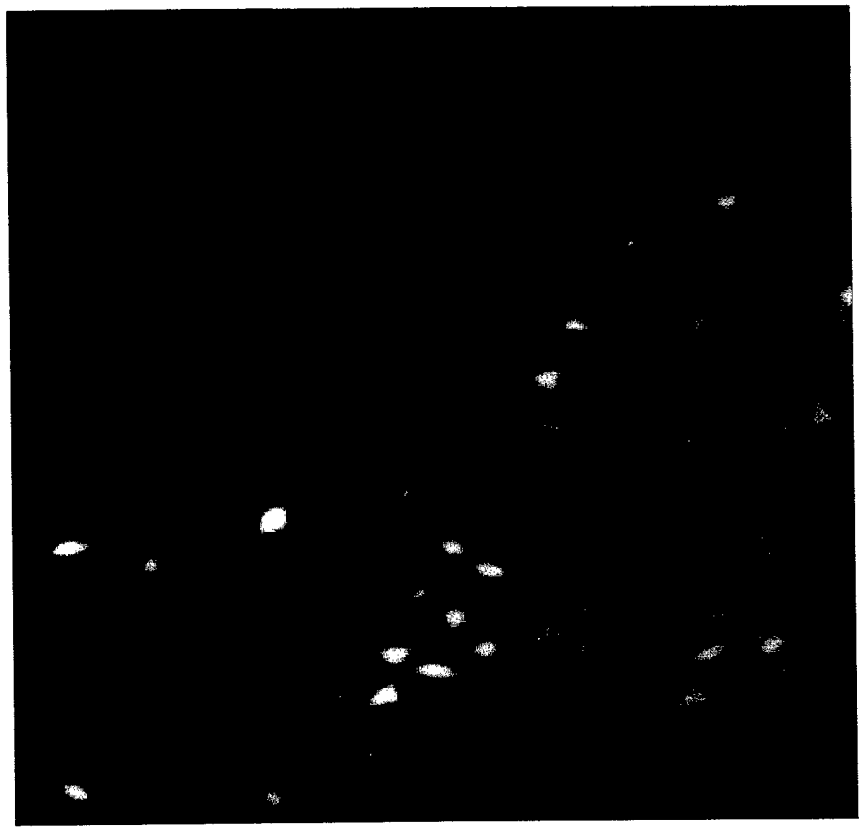


Fig 3: Induction of Cx43 increases EGTA-mediated membrane permeability



Cx43-induced

Non-induced

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Figure Legends:

Figure 1. Connexin 43 is inducible and functional and MDA cells as shown by Western immunoblotting and dye transfer. Left panel: Western blot. Cells were induced to express connexin 43 by withdrawal of doxycycline (-) all were non-induced for 72 hrs. Total proteins were immunoblotted utilizing a connexin 43 antibody. As can be seen, induction resulted in a strong immunoreactive band of appropriate molecular mass. Microinjection studies; center and right panels. Cultures are shown after connexin 43 induction, top panels, or in the non-induced situation, bottom panels. A single cell, indicated by the arrow, was microinjected with Lucifer yellow and spread of dye was followed over the next 10 minutes. As can be seen, after induction approximately 30 cells became fluorescent in the induced situation, whereas no dye spread occurred in the non-induced situation. Similar results were obtained in approximately 30 other injections.

Figure 2. Induced connexin 43 is assemble into junctional plaques. Cells were grown as monolayers and induced to express connexin 43 by 72 hr. of doxycycline withdrawal. Non-induced cells served as controls. Cells were fixed, permeabilized and exposed to connexin 43 antibody and a fluorescene conjugated second antibody. As can be seen, connexin 43 induction resulted in the formation of multiple immunoreactive plaques in regions of cell/cell contact. The studies were conducted at high cell density, thus limiting the quality of the phase-contrast images.

Figure 3. Induction of connexin 43 increases EGTA-mediated membrane permeability. MDA cells were induced, or not induced to express connexin 43, as in figures 1 and 2 above. Cells were then plated at low cell density, so as to limit the formation of inter-cellular junctions, and exposed to EGTA in the presence of Lucifer yellow. After approximately two minutes, cells were returned to normal culture conditions in the absence of Lucifer yellow. Photomicrographs taken under fluorescent optics reveals that connexin 43 expressing cells were much more capable of taking up Lucifer yellow than were cells non-induced to express connexin 43.

Key Research Accomplishments:

1. Creation of five clones in the a human breast carcinoma cell line in which connexin 43 is inducible by doxycycline and in which this gene is functionally active as indicated by formation of functional gap junctions.

2. Demonstration that connexin 43 hemi--channels form in doxycycline-induced cells and can be opened by exposure of cells to low calcium conditions.

Reportable Outcomes:

1. Development of the MDA human breast carcinoma cell line containing a connexin 43 gene inducible by tetracycline.

2. Development of an expression vector containing a mutant connexin 43 gene under the control of a tetracycline-promoter.

3. **Training experiences:** this research has contributed to the research training of two graduate students working towards a Ph.D. in biomedical sciences at the University of Hawaii. This research is

under the direction of the PI of this proposal, Professor Bertram.

Conclusions:

The development of inducible breast cancer cell lines represents an important new development which should allow the unambiguous determination of the function of gap junctional communication and consequences of its down regulation in human breast carcinoma. These cells should be useful not just to achieve the aims of this proposal, but should also allow other investigations into control of proliferation via junctionally transmitted signals.

References:

This proposal has not yet progressed to the point of creating publishable data.

Appendices: None.